Exposure to inhomogeneous static magnetic field beneficially affects allergic inflammation in a murine model

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SUMMARY

Previous observations suggest that static magnetic field (SMF)-exposure acts on living organisms partly through reactive oxygen species (ROS) reactions. In this study, we aimed to define the impact of SMF-exposure on ragweed pollen extract (RWPE)-induced allergic inflammation closely associated with oxidative stress. Inhomogeneous SMF was generated with an apparatus validated previously providing a peak-to-peak magnetic induction of the dominant SMF component 389 mT by 39 T/m lateral gradient in the *in vivo* and *in vitro* experiments, and 192 mT by 19 T/m in the human study at the 3 mm target distance. Effects of SMF-exposure were studied in a murine model of allergic inflammation and also in human provoked skin allergy. We found that even a single 30 min exposure of mice to SMF immediately following intranasal RWPE challenge significantly lowered the increase in the total antioxidant capacity of the airways and decreased allergic inflammation. Repeated (on 3 consecutive days) or prolonged (60 min) exposure to SMF after RWPE challenge decreased the severity of allergic responses more efficiently than a single 30 min treatment. SMF-exposure did not alter ROS production by RWPE under cell-free conditions, while diminished RWPE-induced increase in the ROS levels in A549 epithelial cells. Results of the human skin prick tests indicated that SMF-exposure had no significant direct effect on provoked mast cell degranulation. The beneficial effects of SMF observed are likely due to the mobilization of cellular ROS-eliminating mechanisms rather than direct modulation of ROS production by pollen NAD(P)H oxidases.

Keywords: Allergic inflammation; Oxidative stress; Pollen; Static magnetic field

1. INTRODUCTION

The pollen of short ragweed (*Ambrosia artemisiifolia*) is one of the most common causes of respiratory allergy in North America and Europe [1]. Allergic airway inflammation triggered by ragweed pollen is closely associated with oxidative stress that is defined by disturbance of the equilibrium between reactive oxygen species (ROS) and antioxidant defense mechanisms favoring oxidant species. In pollen-triggered allergic inflammation both exogenous and endogenous sources of ROS have already been identified. Intrinsic NAD(P)H oxidases of intact pollen grains [2], their extracts [3], and subpollen particles of respirable size [4] are known to generate ROS immediately after exposure inducing oxidative stress in the airway epithelium independent of adaptive immune responses [3]. Subsequent oxidative stress in the lungs derives from ROS released by inflammatory cells recruited into the airways several hours after pollen exposure [5,6]. Increased levels of ROS enhance inflammatory responses either directly or via induction of lipid peroxidation [7] and oxidative DNA damage [6]. Thus inhibition of these oxidative insults may be effective in the treatment of pollen-induced allergic symptoms by locally administered antioxidants [8] or by approaches that enhance the lung antioxidant screen [7].

Human subjects and animals respond to the exposure of a wide range of static magnetic fields (SMF). The background of the observed biological effects provoked by SMF-exposure has not yet been fully elucidated, but some of these responses seem to be at least partly mediated through free radical reactions (reviewed in [9]). However, the overall picture of the effects of SMF-exposure on intra- and extracellular ROS levels is ambiguous. There are several studies reporting that moderate SMF in the mT magnetic induction range can influence either the generation or the reduction of ROS in biological systems. The controversial effects of SMF-exposure on reactive radicals observed so far are primarily due to diverse reactions of different cell types and living organisms, various test conditions (homogeneous versus inhomogeneous

SMF, timing and duration of exposure, various magnetic inductions of SMF, etc.), and many disparate methods used for ROS measurement [9].

Currently no published results are available about the effects of SMF-exposure on pollen-induced allergic inflammation. However, it has previously been reported that application of moderate strength SMF for 15 or 30 min immediately after injection of histamine into the hind paws of experimental animals resulted in significant reduction of edema formation [10], whereas exposure to SMF before injection or at the time of maximal edema did not influence edema formation or resolution, respectively.

In the present study, we investigated whether whole-body exposure to a well-defined, inhomogeneous SMF would be able to modify ragweed pollen-induced allergic airway inflammation in a mouse model of allergy. In this model a single intrapulmonary challenge of RWPE-sensitized mice with RWPE was utilized to trigger airway inflammation [11]. For the generation of SMF, we used an apparatus, the parameters of which have previously been described in detail, tested and optimized for small experimental animals [12,13]. We also performed a human study to test whether SMF-exposure would have an effect on provoked skin allergy.

2. MATERIALS AND METHODS

2.1. Generation of inhomogeneous SMF

The inhomogeneous SMF was generated with an exposure system identical to the one previously described (#1 in [12]). Briefly, the device consisted of 2 ferrous matrices (size 170x140 mm) containing 10x10 mm cylindrical neodymium iron boron (NdFeB) N35 grade magnets (B_r=1.20 T). The lateral periodicity was 10 mm. The individual magnets in both matrices were placed next to each other with alternating polarity. Magnets facing each other in the 2 matrices were oriented with opposite polarity. The matrices were fixed in a holder in which the matrices were separated from each other with a distance of 50 mm. This arrangement allowed us to insert a 140x100x46 mm Plexiglas animal cage with ventilation holes on the front and back sides or 6-well cell culture plates into the space that separated the 2 matrices.

In order to test 2 different vertical magnetic induction values and corresponding lateral gradients simultaneously in a single exposure chamber, we planned our *in vitro* experiments with 2 layers. Two 6-well cell culture plates were stacked on top of each other. The SMF at the bottom of the lower culture plate in the stack was denoted by “lower” SMF, the top of the stack was the “upper” SMF. The height of a 6-well cell culture plate (12 mm) defined the distance between the layers. Therefore the actual distance from the magnetic surface was 3 mm for the lower SMF and 15 mm for the upper SMF.

For the skin prick tests a single magnetic matrix of the above exposure system was used (case #3 in [12]). Along the shorter sides (on the contact sites) 2 spacers with soft surface coverage were fixed fulfilling 2 tasks: (i) ease holding the 875 g device on the forearm for 15 min, and (ii) provide a uniform distance of 3 mm between the magnetic (or sham) surface and the skin. On one forearm the magnetic matrix, on the other a sham matrix of identical looks and weight was used simultaneously. A random list prepared prior to the test decided which device

was applied on which forearm. This list was neither revealed to the volunteer, nor to the physician. No metallic object whatsoever was allowed at arm's reach before and during the test.

Typical peak-to-peak vertical magnetic induction values along the axis of a magnet in the isocenter of the generator in the *in vivo* and *in vitro* experiments were 389.46±0.1 and 2.97±0.1 mT, whereas the lateral gradient values between the 2 neighboring local extremes were 39.25 and 0.22 T/m at 3 and 15 mm from the surfaces of matrices, respectively (case #1 in [12]). Values of the SMF used in the skin prick test were: 192.28±0.1 mT by 18.89 T/m lateral gradient at 3 mm from the magnet [12]. Horizontal components of the magnetic induction were one order of magnitude smaller than those of the vertical ones and were regarded as stray field components. The dosimetric measurements were carried out by a gaussmeter (Lake Shore Cryotronics, Model 420, Westerville, OH, USA).

As lighting is an important issue for both the *in vitro* tests and the *in vivo* mouse experiments we took special care of balancing the SMF- and the sham-exposed situations. In short, the walls of both SMF- and sham-exposed cell culture plates were transparent to visible light, but the exposures were carried out in total darkness. The Plexiglas animal cages were covered with an opaque material on top and bottom and on 2 opposite sides. The cages in the SMF-exposure chamber were identically illuminated as under sham-exposure: through the front and back sides of the cages. Illumination was provided by fluorescent lights (Model L58W/640, Osram, Munich, Germany) from above during the experiments. The fluorescent lamps generated a basically horizontal scattered light in the shaded area of the Plexiglas cages with intensity between 6 and 15 mW/m^2 as previously described [14]. The applied light sources emit light with several wavelength peaks between 403 and 710 nm. The calculated integral light intensity maximum corresponds to $3.9x10^{16}$ photons/s/m^2, which is below the intensity threshold for altering behavioral responses in mice in SMF-shielded environments [15]. The illumination

conditions inside the cage were basically independent of the location of the mouse within the cage. Horizontal light gradients did not occur between front and back sides. We carried out the *in vivo* animal experiments in the same period of time of the day, between 8 and 12 a.m. being aware that rodents are subject to the circadian cycle in almost all areas of their life [16]. Due to the closed design of the Plexiglas animal cages, ventilation through the cages was restricted to the front-back direction for both SMF- and sham-exposed animals. Temperature and relative humidity were kept constant within prescribed limits during the experiments. In the *in vitro* tests, all cell culture plates were covered and incubated at 22°C during SMF- and sham-exposures.

Experiments were conducted in Debrecen, Hungary (47˚31′ N, 21˚38′ E). The background SMF in the lab was the geomagnetic field, the horizontal components of this field needed to be taken into account (total magnetic induction of 21,138.75 nT, grown to 21,160.79 nT during the time period of the studies [17]). Although the geomagnetic field itself is known to have the capacity to affect living organisms (e.g., migrating animals), in the present studies we only looked for the differences in the biological response to exposure to an artificial external SMF 7 orders of magnitude stronger than that of Earth. This stronger SMF was simply superimposed for the magnetic background in case of SMF-exposed objects.

2.2. Participants in the skin prick test, ethics

The study population consisted of 62 volunteers (21 males and 41 females, age between 22 and 50 years). Exclusion criteria of the study were: pregnancy or lactating, using medication for allergies, or abnormal spirometry test results. All participants provided written informed consent. The placebo-controlled, double-blind, randomized human study was approved by the Regional and Institutional Ethics Committee of the University of Debrecen, Medical and Health Science Center (Debrecen, Hungary, # DE OEC RKEB/IKEB 3854-2013) and conducted at the

Department of Pulmonology of the University of Debrecen under the supervision of a specialist. The tests were carried out in April-May 2013. No regulation for the use of permanent magnetic devices is available in the European Union below 8 T magnetic induction. For potential risks of SMF-exposure, see the report of the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR 2009). Colbert and co-workers proposed a standardization of the description of clinical study reports including SMF [18]. Table I contains our corresponding data.

2.3. Animals, ethics, groups in the *in vivo* tests

Experiments were carried out on 8 week old female Balb/c mice (Charles River, Wilmington, MA, USA). Animals were randomly divided into experimental groups. For SMF-exposure 3 animals were put into the perforated cage at a time, then the cage with the animals was inserted into the exposure chamber of the magnetic device for 30 or 60 min. Sham-exposure was carried out by placing the 3 animals in identical cages without inserting the cage in the exposure chamber. Identical SMF-exposure was shown not to cause any change in the anxiety and locomotive behavior of mice [19].

Care and handling of animals followed the Helsinki Declaration, European Union regulations and adhered to the guidelines of the Committee for Research and Ethical Issues of IASP. All animal study protocols were approved by the Animal Care and Protection Committee at the University of Debrecen (#7/2011/DE MAB). Animals were maintained in the pathogen-free animal facility of the University of Debrecen.

All animals were sensitized with 2 intraperitoneal administrations (on day 0 and 4) of 150 μg/injection endotoxin-free RWPE (Greer Laboratories, Lenoir, NC, USA), combined in a 3:1 ratio with alum adjuvant (Pierce Laboratories, Rockford, IL, USA) as previously described [3]. On day 11, mice were challenged intranasally with 100 μg RWPE dissolved in 60 μl of phosphate

buffered saline (PBS, PAA Laboratories, Pasching, Austria) or same volumes of PBS as a vehicle. On day 14, mice were euthanized and allergic inflammation was evaluated. To test whether SMF-exposure had an effect on allergic airway responses of the treated animals and to determine the optimal timing of exposure and the size of animal groups required for reasonable statistical considerations we first performed a pilot study.

Animal groups in the pilot study

Group A Animals (*n*=5) were exposed to sham field (no SMF) for 30 min a day from day 0 through day 13 (sham treatment on day 11 was performed immediately after intranasal RWPE challenge)

Group B Animals (*n*=5) were exposed to SMF for 30 min a day from day 0 through day 11 (last treatment was performed 6 h prior to intranasal RWPE challenge)

Group C Animals (*n*=5) were exposed to SMF for 30 min a day from day 11 through day 13 (treatment on day 11 was performed immediately after intranasal RWPE challenge)

Group D Animals (*n*=5) were exposed to SMF for 30 min a day from day 0 through day 13 (treatment on day 11 was performed immediately after intranasal RWPE challenge)

Group E Animals (*n*=3) were exposed to SMF for 30 min a day from day 0 through 13 (treatment on day 11 was performed immediately after intranasal PBS challenge)

Animal groups in the full test

Group I Animals (*n*=19) were exposed to sham field (no SMF) for 30 min a day from day 11 through day 13 (treatment on day 11 was performed immediately after intranasal RWPE challenge)

Group II Animals (*n*=21) were exposed to SMF for 30 min only on day 11 immediately after intranasal RWPE challenge

Group III Animals (*n*=21) were exposed to SMF for 30 min a day from day 11 through day 13 (treatment on day 11 was performed at 6 h after intranasal RWPE challenge)

Group IV Animals (*n*=8) were exposed to SMF for 60 min a day from day 11 through day 13 (treatment on day 11 was performed at 6 h after intranasal RWPE challenge)

Group V Animals (*n*=8) were exposed to SMF for 30 min a day from day 11 through day 13 (treatment on day 11 was performed at 6 h after intranasal PBS challenge)

2.4. Evaluation of allergic inflammation in mice

Inflammatory cell infiltration into the airways was assessed by the analysis of the bronchoalveolar lavage fluid (BALF) at 72 h after allergen challenge as previously described [4]. To collect BALF animals were euthanized and their tracheas were cannulated. Lavage was performed with 2 aliquots of 0.7 ml of ice cold PBS (pH 7.3). The BALF samples were centrifuged (400g for 10 min at 4°C), the supernatants were removed and stored at -80°C for further analysis. Total cell counts in the BALF were determined from an aliquot of the cell suspension. Eosinophils, neutrophils, lymphocytes, and macrophages on Wright-Giemsa-stained cyto-centrifuge preparations were enumerated by counting at least 400 cells. Assessment of lung histology was carried out as previously described [3]. Briefly, the lungs were fixed following BALF collection by inflating with formalin. Coronal sections of the formalin-fixed lungs were stained with hematoxylin and eosin for estimating inflammation in subepithelial regions or periodic acid-Schiff stain for assessing the abundance of mucin producing cells. Stained sections were analyzed by using a Photometrics CoolSNAP Fx CCD (Tucson, AZ, USA) digital camera mounted on a Nikon Eclipse TE 200 (Tokyo, Japan) fluorescent microscope.

2.5. Measurement of mucin levels in BALF samples from mice

MUC5AC levels in BALF were assessed by enzyme-linked immunosorbent assay (ELISA) as described previously [20]. In brief, serial dilutions of BALF were incubated at 37°C in triplicate 96-well plates until dry. Plates were blocked with 2% bovine serum albumin (BSA) in PBS for 1 h and incubated with 50 µl (1:10,000 dilutions) of biotin-conjugated mouse monoclonal MUC5AC antibody (Lab Vision, Fremont, CA, USA). After 60 min the plates were washed with PBS-BSA and further incubated with streptavidin-horseradish peroxidase goat anti-mouse IgG conjugate (1:10,000) for 1 h. Plates were washed (twice with PBS-BSA) and were incubated with peroxidase substrate (3,3',5,5'-tetramethylbenzidine) to obtain the colorimetric product, which was quantified at 450 nm. Results were expressed as endpoint titers [21].

2.6. Cell cultures

The A549 human bronchial epithelial cells (American Type Culture Collection, Manassas, VA, USA) were cultured at 37°C in a humidified atmosphere with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) with Glutamax (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen), penicillin (100 U/mL), and streptomycin (100 µg/mL).

2.7. Measurement of ROS generated by RWPE under cell-free conditions

2'-7'-Dihydro-dichlorofluorescein diacetate (H_2DCF-DA, Molecular Probes, Eugene, OR, USA) was used to detect ROS production by RWPE. Ragweed pollen proteins (100 µg/ml) and 50 µM H_2DCF-DA were incubated in PBS containing 100 µM nicotinamide adenine dinucleotide phosphate-oxidase (NADPH, Sigma-Aldrich, St. Louis, MO, USA) in 2 ml final volume in 6-well plates (TPP, Trasadingen, Switzerland). Regular PBS solution containing 50 µM H_2DCF-DA and

100 μM NADPH was applied as control. Plates were exposed to sham field or to SMF (either at lower or upper position, see in Magnetic device section) for 30 min and changes in the dichlorofluorescein (DCF) fluorescence intensity were determined using a Synergy HT micro plate reader (Bio-Tek Instruments, Winooski, VT, USA) at 485 nm excitation and 528 nm emission.

2.8. Assessment of intracellular ROS levels in cultured epithelial cells

A549 cells grown to 70% confluence in 6-well plates were loaded with 50 μM H_2DCF-DA (Molecular Probes) at 37°C for 15 min. After removal of the excess probe, cells were treated with PBS containing NADPH (100 μM) or RWPE (100 μg/ml) plus NADPH (100 μM). Immediately following this treatment the cell cultures were sham- or SMF-exposed for 30 min either at lower or upper position. Changes in DCF fluorescence intensity were assessed in a Synergy HT micro plate reader (Bio-Tek Instruments) at 488 nm excitation and 530 nm emission.

2.9. Measurement of total antioxidant capacity of murine airways

To measure total antioxidant capacity of the airways, naive mice were intranasally challenged with RWPE (100 μg dissolved in 60 μl PBS) or with identical volumes of PBS immediately preceding the exposure to SMF or sham field for 30 min. Bronchoalveolar lavage was performed 15 min after treatment as described above. The BALF samples were centrifuged (400g for 10 min at 4°C) and then the total antioxidant potential of BALF samples was measured in the supernatants spectrophotometrically at 570 nm by using a Total Antioxidant Capacity Assay Kit (Abcam, Cambridge, UK). A standard of known 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, included in the kit) concentration was used to create a calibration curve (R^2=0.999) and the results of the assay were expressed as nmol/μL Trolox equivalents.

2.10. Skin prick test on human volunteers

The tests were performed simultaneously on both inner forearms of the volunteers. All participants were tested with positive control (histamine), negative control (saline) and with 4 aeroallergens (house dust mite, cat fur, mixed grass pollen, and ragweed pollen; all from ALK-Abello, Hørsholm, Denmark). In order to minimize the variability of the results (the volume of administered test samples and the depth of scrapes) Multi-test II applicators (Lincoln Diagnostics, Decatur, IL, USA) were used. Immediately after introduction of the identical test materials into the skin of both inner forearms, one forearm of the volunteers was exposed to SMF while the other one was exposed to sham field. The wheal reaction was measured immediately after a 15 min exposure period.

Finally, 12 test results out of 62 were excluded from the statistical assessment. The reason was one of the followings: (i) wheal of 3 mm or bigger in diameter response to the negative control indicating severe dermatographism (n=4); (ii) nonidentical responses to the negative control on the left and right forearms (n=8). The remaining sample population contained 15 males and 35 females of average age 30.9 years.

2.11. Statistical analysis

The normal distribution of the measured data was checked by using Kolmogorov-Smirnov tests in all measurements: in the *in vivo* experiments, in the *in vitro* tests, and also in the human trial. In case of normal sample populations one-way ANOVA was used to reveal significant differences between multiple groups. For *post hoc* analysis Games-Howell tests were applied between pairs of data series, partly because this test is insensitive to unbalanced data size. For non-normal populations (like the endpoint titer values) Kruskal-Wallis test was used for multiple group analysis and then Mann-Whitney tests were carried out for the *post hoc* binary

comparisons. Probabilities (p) of less than or equal to 0.05 between groups were considered to be statistically significant at the 95% confidence level. Probabilities lower than 0.001 are not shown numerically in the text. Numerical values presented in the figures indicate mean values ± standard errors of mean (SEM). Statistical analyses were made using Excel (Microsoft) and XLStat 7.5 softwares (Addinsoft).

We defined the effect in percent meaning M=1-(value in one group)/(value in other group). By such a definition, the effect can be negative and can exceed 100% in absolute value.

3. RESULTS

3.1. SMF-exposure did not affect the sensitization phase of pollen allergy in mice

In the pilot test mice were divided into 5 groups (A-E, see Materials and methods and Fig. 1a) to investigate whether SMF-exposure has an effect on the sensitization or elicitation phase of RWPE-induced allergic reactions. Daily exposure to SMF during the 11-day long sensitization phase prior the intranasal RWPE challenge did not affect the accumulation of eosinophils in the BALF compared to sham-exposure (see Group B and A in Fig. 2). On the contrary, a remarkable but statistically not significant decrease in eosinophil count was detected, when mice were exposed to SMF on 3 consecutive days (days 11-13) after the intranasal challenge (see Group C in Fig. 2). Daily exposure of RWPE-challenged mice to SMF during the 13-day long experimental period also decreased eosinophil counts in the BALF compartment as compared to RWPE-challenged, sham-exposed mice (see Group D and A in Fig. 2). These observations indicated that SMF-exposure did not affect the sensitization phase of the allergic responses and prompted us to examine the effect of SMF-exposure in the elicitation phase in more detail and to increase the number of animals per group for a higher statistical power.

3.2. Exposure to SMF during the elicitation phase decreased allergic airway inflammation in RWPE-sensitized mice

In the full experimental series mice were divided into 5 groups (I-V), were sensitized with RWPE, challenged with RWPE or PBS, and exposed to SMF or sham field (Fig. 1b). Exposure to SMF for a single 30 min time period immediately after the intranasal challenge on day 11 induced a moderate, but significant decrease in total cell counts in the BALF (Fig. 3a) (M=14.90%, p=0.003), strongly and significantly decreased number of eosinophils in BALF (Fig. 3b) (M=33.81%, p<0.001), and lowered the infiltration of inflammatory cells into the

subepithelial area of the airways (Fig. 3c) compared to RWPE-challenged, sham-exposed animals. A single SMF-exposure also decreased MUC5AC levels in BALF to some extent (Fig. 4a) (M=14.63%, p=0.113) and also decreased epithelial cell metaplasia in the airways (Fig. 4b) as compared to Group I. Significantly lower numbers of total cells (Fig. 3a) (M=21.48%, p<0.001) and eosinophils (Fig. 3b) (M=26.50%, p<0.001) in the BALF together with decreased accumulation of inflammatory cells in the subepithelial area were also detected (Fig. 3c) upon exposure to SMF for 30 min on 3 consecutive days (days 11-13) following RWPE challenge. This 3x30 min SMF-exposure significantly reduced MUC5AC levels (M=19.51%, p=0.035) in BALF (Fig. 4a) and markedly lessened epithelial cell metaplasia in the airways (Fig. 4b) as compared to sham-exposed animals. Prolonged exposure to SMF (60 min a day) on 3 consecutive days (days 11-13) after RWPE challenge further decreased the total cell (M=26.67%, p<0.001) (Fig. 3a) and eosinophil (M=57.70%, p<0.001) (Fig. 3b) numbers in the BALF, and lowered inflammatory cell accumulation in the subepithelial regions of the airways as well (Fig. 3c). Prolonged SMF-exposure was trend-wise more effective to abate total (M=6.60%, p=0.207) and significantly more effective in lessening of eosinophil cell (M=42.45%, p<0.001) influx into the airways than exposures for 3x30 min (Fig. 3b). Furthermore, the 3x60 min exposure to SMF was more effective in decreasing MUC5AC levels in the BALF (M=36.36%, p=0.019) (Fig. 4a) and to lower epithelial cell metaplasia in the airways (Fig. 4b) than the 3x30 min treatment. These results suggest that even a single 30 min exposure to SMF immediately after intranasal allergen challenge is able to decrease airway inflammation. In addition, increased doses of SMF-exposure either by increasing exposure time period (more days) or by extended duration of individual treatments (60 min instead of 30 min) can further decrease the severity of allergic inflammation in the lung.

3.3. SMF-exposure did not alter ROS production by RWPE under cell-free conditions, while diminished RWPE-induced increase in the ROS levels in cultured epithelial cells

To investigate the mechanism behind the observed inhibitory effect of SMF-exposure on allergic airway inflammation, we tested whether SMF-exposure was able to decrease the ROS production by RWPE under cell-free conditions. In accordance with our previous work [3], sham-exposed RWPE converted the redox-sensitive H_2DCF-DA into dichlorofluorescein (DCF) leading to 75 times higher fluorescence intensity ($p<0.001$) than that of PBS control (Fig. 5). However, exposure to SMF for 30 min either at lower or upper position (see in Magnetic device section) did not alter DCF fluorescence signals induced by RWPE (Fig. 5a). Next, we studied the effect of SMF-exposure on intracellular ROS levels in cultured airway epithelial cells. A549 cells loaded with H_2DCF-DA were treated with PBS or RWPE and immediately following this treatment the cell cultures were sham- or SMF-exposed for 30 min at lower or upper position (see in Magnetic device section). Addition of RWPE to A549 cells induced a 3.6-fold increase ($p<0.001$) in intracellular DCF fluorescence signals compared to PBS treatment (Fig. 5b). The increase in intracellular ROS levels could significantly be diminished (M_{max}=20.57% at 135 min, p=0.002), when the cells were exposed to SMF at the lower position and also, to a smaller extent, when they were in the upper position (M_{max}=9.34% at 135 min, p=0.034) (Fig. 5b). Exposure to SMF, either at lower or upper position, did not cause significant changes in levels of intrinsic ROS in PBS-treated cells (Fig. 5b). These observations suggest that although SMF is not able to decrease ROS production by RWPE directly or lessen the life-span of ROS under cell-free conditions, it still may inhibit ROS entry into living cells or promote elimination of ROS by cellular mechanisms.

3.4. Exposure of mice to SMF immediately after intranasal RWPE challenge lowered the increase in the total antioxidant capacity of the airways

We have previously demonstrated that intranasal RWPE treatment rapidly increases ROS levels in the lungs of experimental animals prior to the recruitment of inflammatory cells [3]. Based on the results of our cell culture studies we sought to test, whether SMF-exposure could decrease ROS levels in the airways of RWPE-treated mice. To do so, total antioxidant capacity of the BALF samples collected from naive mice challenged intranasally with RWPE or PBS and exposed to SMF or sham field was determined. Intranasal challenge with RWPE induced a nearly 3-fold, statistically significant increase in the antioxidant capacity of the BALF samples when compared to PBS challenge ($p<0.001$) (Fig. 6). Immediate exposure to SMF for 30 min after intranasal challenge significantly lowered ($M=20.07\%$, $p<0.001$) the increase in the total antioxidant capacity of the airways induced by RWPE treatment (Fig. 6). These findings suggest that effects of SMF-exposure on allergic inflammation are mediated at least partially by the modulation of ROS levels in the airways.

3.5. SMF-exposure had no effect on provoked mast cell degranulation in human skin

Several lines of evidence indicate that ROS play an important role in the regulation of various mast cell responses [22-24]. To reveal the direct effects of SMF-exposure on mast cell degranulation, human skin prick tests were performed. A statistically significant effect of the SMF-exposure decreasing edema diameter ($M=5.29\%$, $p=0.016$, $n=50$) could only be detected in case of the positive control, histamine (Fig. 7). Although all allergens (cat fur, $n=17$; grasses, $n=13$; RWPE, $n=25$), but house dust mite ($n=14$) provoked edema showed a tendency to decrease the diameter upon SMF-exposure (Fig. 7). These results indicate that SMF-exposure can result in significant reduction of histamine-induced edema formation, while it performs only a weak direct impact on provoked mast cell degranulation.

4. DISCUSSION

There has been a significantly increased prevalence of allergic inflammatory diseases over the last few decades, which appears to be related to changes in the environment that affect susceptible individuals, both in the induction and worsening of established disease [25]. The clinical complication of airway inflammation and subsequent airway hyper-responsiveness are the leading causes of morbidity and mortality in critically ill patients. While many aspects of pathogenesis of allergic inflammation are well-defined, most of the treatments are symptomatic. In this study, we report that exposures to SMF significantly lowered RWPE challenge-induced allergic inflammation in a murine model. Although the precise mechanism by which SMF mediates beneficial effects needs to be elucidated, our novel observation may provide a hint on a future non-invasive therapeutic modality for treatment of human allergic airway inflammation.

We found that the effects of SMF-exposure on allergic inflammation were mediated at least partially by decreasing ROS levels in the airways. Immune responses leading to allergic inflammation can be divided into sensitization and elicitation phases. In the sensitization phase activated antigen presenting cells, such as dendritic cells present allergens to naive T cells, which results in the generation of Th2 cells producing cytokines essential for allergen-specific IgE generation by B cells. We have previously demonstrated that ragweed pollen NAD(P)H oxidases increase the intracellular ROS levels in human monocyte-derived dendritic cells leading to their maturation and activation [26,27]. These observations suggest that pollen exposure-induced oxidative stress may participate in the initiation of adaptive immune responses to pollen antigens. Despite this assumption, the pilot experiment of this study revealed that daily SMF-exposure during the sensitization phase of RWPE-induced allergic reactions did not modify the intensity of the developing airway inflammation. In the experimental allergy model we used, mice were injected intraperitoneally with a mixture of RWPE and alum to elicit allergic sensitization. It is

widely accepted that alum acts as an adjuvant to activate the intracellular stress sensor inflammasomes [28,29] and ROS are required for inflammasome activation [30]. However, the critical role of alum as an adjuvant to activate inflammasomes has been controversial, as alum can exert adjuvanticity also in mice deficient of inflammasomes [31,32]. A recent study revealed that alum-induced adjuvant effects are dependent on inducible heat shock protein 70 (hsp70) [33]. Although no data are available on the effects of SMF-exposure on hsp70 expression in murine antigen presenting cells, it has previously been reported that 100 mT SMF-exposure has no significant effect on hsp70 production in NIH3T3 cells [34]. These observations indicate that the adjuvant effects of alum on hsp70 overcome the ROS-mediated activating signals in antigen presenting cells during the sensitization.

Reactive radicals generated by pollen NAD(P)H oxidases induce oxidative stress in the airways within minutes of exposure [3]. Oxidative insult is able to disrupt airway epithelial cell tight junctions [35] thus promoting the interaction of allergens with mast cells and also contributing to the recruitment of inflammatory cells in the airways [3]. Challenge with RWPE induces an initial neutrophil recruitment that is followed by eosinophil influx [36] and both cell types are known to contribute to oxidative stress during allergic inflammation [37]. The neutrophil influx starts at 4 h and peaks at 24 h following RWPE challenge [8] and as a response to oxidative stress elevated antioxidant capacity of the airways becomes detectable [38]. We have found that a single SMF-exposure immediately after intranasal RWPE challenge down modulated the increase in antioxidant capacity and also lowered allergic inflammation. These findings suggest that SMF-exposure is able to attenuate initial oxidative stress elicited by pollen NAD(P)H oxidases. In addition, repetitive exposure to SMF on 3 consecutive days, starting at 6 h after intranasal RWPE challenge by the time the initial oxidative burst had been abolished [8] also inhibited allergic airway inflammation. In a previous study we have demonstrated that

scavenging RWPE-generated ROS was able to prevent allergic inflammation in the airways by coadministering antioxidants [8]. However, scavenging ROS generated by neutrophils recruited at 4 and 24 h failed to do so [8]. These observations raise the possibility that SMF-exposure either has distinct effects on ROS production and elimination than those of antioxidants (ascorbic acid and N-acetyl cysteine), or it can inhibit the recruitment of inflammatory cells by ROS-independent mechanism(s). Indeed, it has previously been reported that isolated neutrophils, which appear to be highly sensitive to both static and alternating magnetic fields [39], under the exposure to strong SMF (0.6-2 T) generated significantly less superoxide anions than detected in controls [40]. Furthermore, in a recent study a significant decline in ROS production by human peripheral blood neutrophils has been shown after 15 min exposure to SMF (~60 mT), while a longer incubation time (45 min) caused a reverse phenomenon [41]. The authors conclude that the SMF-exposure may directly modulate the activity of neutrophil NADPH oxidases and they highlight the importance of proper adjustment of exposure time to SMF for any potential therapeutic applications.

SMF-exposure was found not to be able to modify ROS production by NAD(P)H oxidases in RWPE in cell-free solution. Concerning magnetic spin effects, SMF-exposure can alter those biochemical reactions that involve more than one unpaired electron. It has been shown that the enzyme activity of B_{12}-dependent ethanolamine ammonia lyase changes with SMF-exposure of 100 mT [42]. Experiments have also been carried out with the heme enzymes, horseradish peroxidase, and cytochrome P-450 (as reviewed in [9]). The exact components, structure, and the mechanism of the enzymatic reaction of pollen NAD(P)H oxidases have not been fully determined yet; therefore, no previous studies have investigated the parameters of superoxide generation by these enzymes under SMF-exposure.

In our cell culture experiments SMF-exposure dampened the increase in intracellular ROS levels in RWPE-treated, cultured A549 epithelial cells. Pollen NAD(P)H oxidases generate superoxide anions by transferring electrons from NADPH or NADH to molecular oxygen [3]. Superoxide anions in living cells are converted by superoxide dismutase to hydrogen peroxide molecules, which are eliminated by catalases and glutathione peroxidases (reviewed in [43]). Although the molecular mechanisms of the antioxidant effects of SMF-exposure in our cell culture experiments remain to be elucidated, our observations are consistent with a recent study demonstrating that SMF-exposure decreases externally induced oxidative stress modulating activities of antioxidant enzymes including superoxide dismutase, glutathione peroxidase, and catalase in mice fibroblasts [44].

In order to interact with mast cells the allergen must penetrate into the epithelium during exposure to natural pollen- or subpollen particles. To reveal the direct effects of SMF-exposure on mast cell degranulation, skin prick tests were performed in which small scratches allow the allergens to enter the skin and activate mast cells instead of relying on enzyme activities present in allergy provoking material. In these tests histamine was utilized as positive control, because its injection into the skin by prick technique mimics the allergen-induced edema formation. The results of our human study showed that SMF-exposure significantly decreased edema diameter in response to histamine in agreement with data obtained in an animal model, in which application of SMF-exposure of moderate field strength (5-100 mT) for 15 or 30 min immediately following histamine injection into hind paws resulted in significant edema reduction [10]. Based on these pharmacological experiments the authors proposed that SMF-exposure may activate L-type Ca^{2+} channels in vascular smooth muscle cells that results in increased intracellular Ca^{2+} levels and induces constriction thus limiting edema formation [10]. Although their work provided a rigorous scientific evaluation of the physiological effects of SMF-exposure on edema in a rat model, it

remains to be determined whether SMF-exposure changes vascular tone and/or influences microvascular permeability in human tissues (the possibilities of which were discussed in [45]). Our findings that SMF-exposure was not able to significantly decrease edema formation, triggered by extracts of ragweed and grass pollens both possessing NAD(P)H oxidase activity, can be explained by our previous observations that ROS generated by pollen grains-associated NAD(P)H oxidase have no direct impact on IgE-mediated mast cell degranulation [24]. On the other hand, while histamine is the major mediator of edema formation, during degranulation, mast cells release other compounds such as tryptase, cathepsin G, tumor necrosis factor and vascular endothelial growth factor, which are able to increase vascular permeability (reviewed in [46]). The effects of these vasoactive mediators could be found behind the phenomenon that the size of the edema usually does not correlate with the concentration of histamine released from activated mast cells, and some patients show no significant histamine release during the immediate phase of allergy as assessed by the microdialysis technique [47]. Based on these observations, varying and/or low levels of released histamine also could be an explanation for the weak impact of SMF-exposure on provoked mast cell degranulation.

Although the same magnetic matrix/matrices were used for SMF generation in all experiments, some potential differences in the SMF-induced effects acting at the target site can be predicted. Namely, the self-motion of an electrically charged object (like mouse) in an external SMF as occurs in the *in vivo* experiments may generate motion-induced currents in its own body. Such a current can influence the distribution of specific cells in the lung, but hardly any induced current can occur in cells of the *in vitro* experiments. In the human trial induced currents can be generated without self-motion of the forearm in the exposure chamber of the SMF generator. The motion of electrically charged cells within the blood vessels of the forearm manifests a source of induction. This motion-induced effect may influence the observed biological responses to SMF-

exposure. We have a dual solution to this argument. First, no results were compared between different types of measurements (*in vitro*, *in vivo*, and human). Second, the effect of induced currents under specific SMF-exposure conditions provided here for the *in vivo* experiments has been estimated not to affect the physiology of mice significantly as previously discussed [48]. Even if it did, this fact would not counteract the merit of SMF-exposure in the clinical application, since homogeneous magnetic induction up to 8 T is accepted not to influence blood circulation [49] and this complies with the present guidelines of magnetic resonance imaging (MRI) safety [50].

MRI was agreed not to be regarded as a purely diagnostic device [19,51], its SMF component (B_0) exerts finite forces in the living body and accordingly, invokes such responses that may be observable, perceptible through changes in the (patho)physiology. While the direction of the dominant component of the SMF (B_0) within the tunnel of a conventional MRI is *superior-inferior*, mice in our *in vivo* experiments were exposed to a *ventral-dorsal* SMF in the inhomogeneous SMF generator. Fringe SMF around functioning MRI devices are typically gradient SMF and their magnetic induction might exceed that of the geomagnetic field by orders of magnitude. Furthermore, magnetic induction components of this fringe SMF are greater than those of Earth in all directions. Focusing on the internal side of an MRI, our experimental arrangement resembles more to an open field MRI. However, while humans in and around MRI devices are exposed to SMF with their whole body, only local exposure to a strong inhomogeneous SMF was used in the present human trial. Although the relevance of our study to human allergic airway inflammation remains incidental, our data suggest that effects of the SMF gradients around MRI systems, mainly around open field MRI, should be more carefully investigated, because exposure to SMF including stray field components of MRI may have beneficial effects on pollen-induced allergic conditions.

ACKNOWLEDGEMENTS

This work was supported by the Hungarian Scientific Research Fund (NK-101538 to E.R., K-73347 and K-109595 to A.B.), the European Union and the State of Hungary, co-financed by the European Social Fund in the framework of TÁMOP 4.2.4.A/2-11-1-2012-0001 'National Excellence Program' (A.Cs. and K.P.), the TÁMOP 4.2.2/B-10/1-2010-0024 project (K.P.) and the TÁMOP-4.2.2.A-11/1/KONV-2012-0023 project (E.R. and A.B.). J.F.L was partially supported by the TÁMOP-4.2.2.C-11/1/KONV-2012-0001 project. The project is implemented through the New Hungary Development Plan. A.B. was also supported by the János Bolyai Fellowship from the Hungarian Academy of Sciences and by the Faculty of Medicine Research Fund from the University of Debrecen (Bridging Fund 2012). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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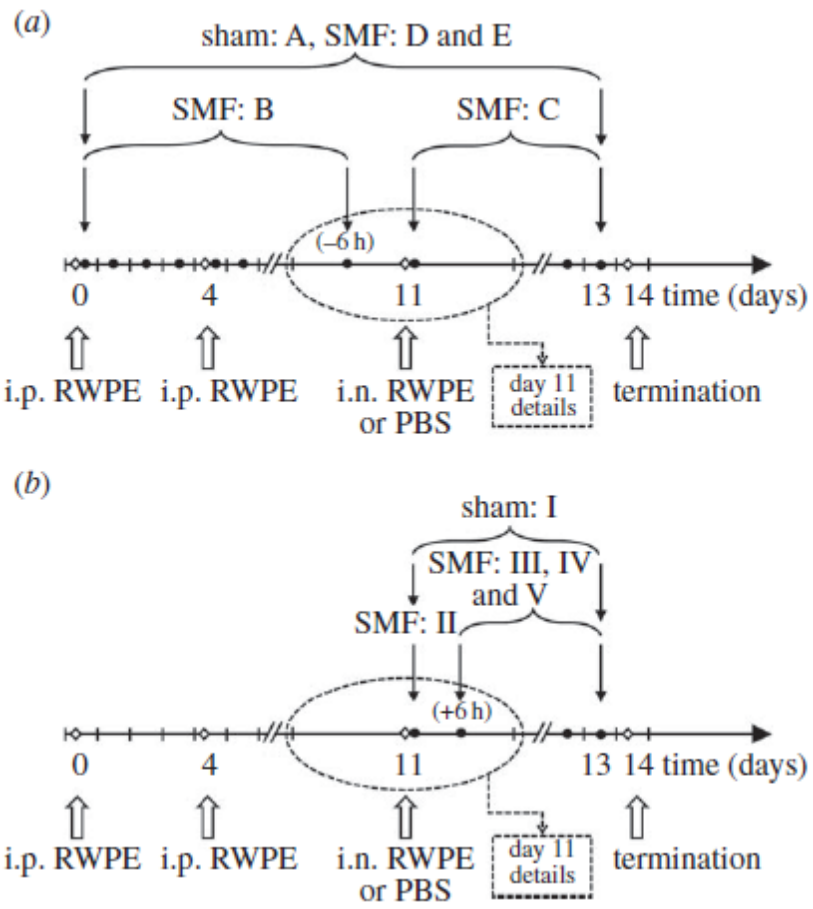

FIGURE 1. Experimental protocols for the pilot study (**a**) and the full test (**b**). Mice were divided into 5 groups both in the pilot study (A-E) and in the full test (I-V). Black dots indicate the dates of static magnetic field (SMF)-exposures and open squares represent the dates of intraperitoneal (i.p.) and intranasal (i.n.) administration of ragweed pollen extract (RWPE) or phosphate buffered saline (PBS) or termination of the experiment, respectively.

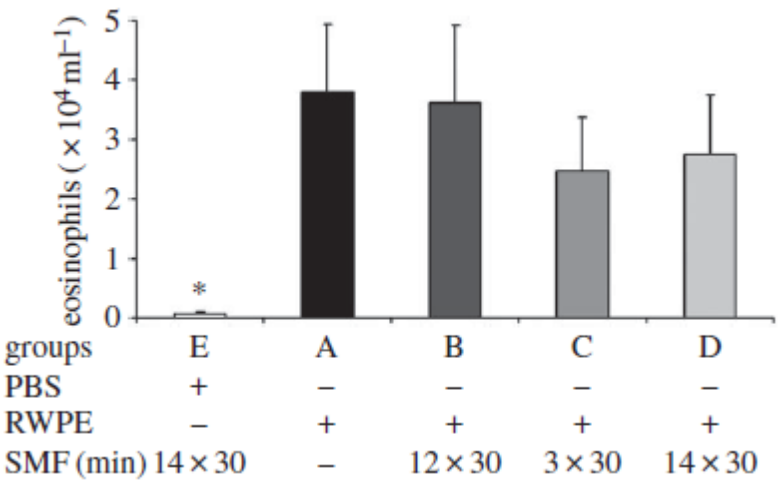

FIGURE 2. SMF-exposure does not affect the sensitization phase of the allergic responses. RWPE or PBS challenged, sensitized mice were exposed to SMF or sham field for 30 min daily during sensitization (Group B) or elicitation (Group C) phase only or the whole period of the experiment (Group A, D, E). Three days after challenge bronchoalveolar lavage (BAL) was performed and lavage samples were examined for eosinophil cell counts. Results are presented as means ± SEM. * $p < 0.05$ *vs*. RWPE challenged, sensitized mice exposed to sham field.

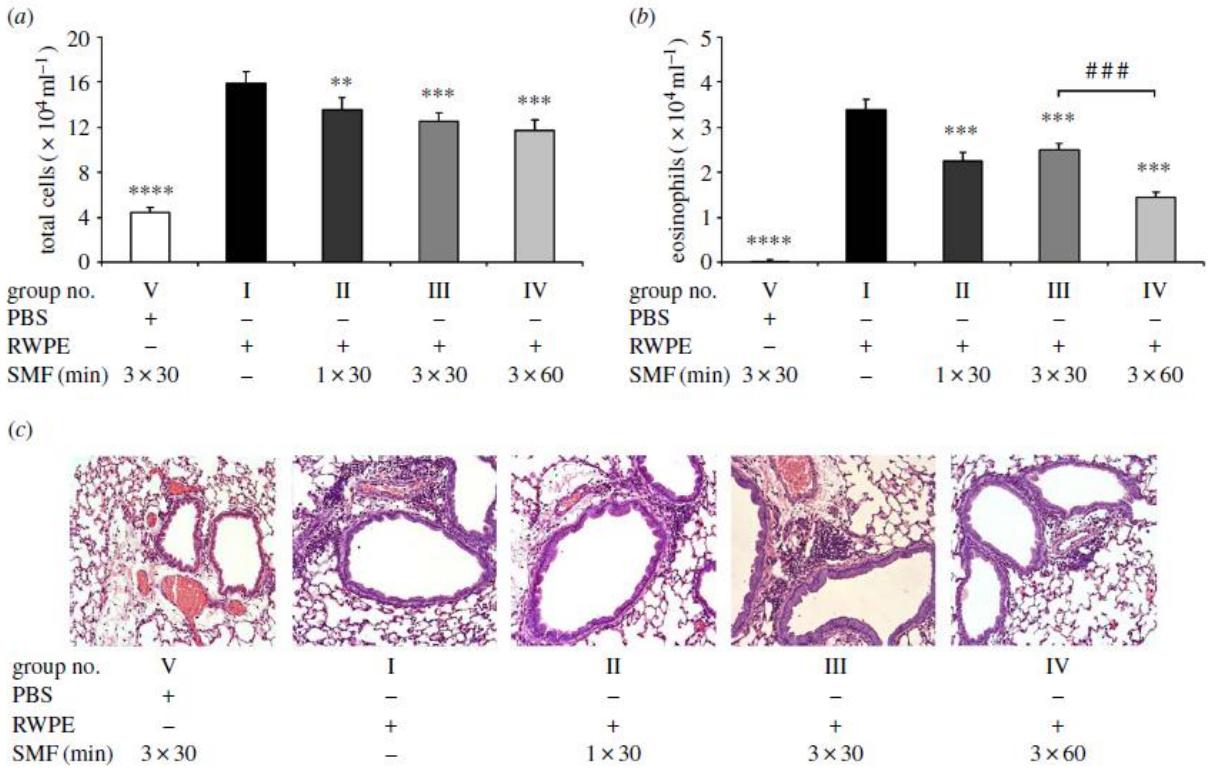

FIGURE 3. Exposure to SMF reduces RWPE-induced allergic airway inflammation. Sensitized mice were challenged with PBS or RWPE and exposed to SMF or sham field. Three days after challenge BAL was performed and lavage samples were examined for total (**a**) and eosinophil (**b**) cell counts. (**c**) Hematoxylin and eosin staining of formalin-fixed lung sections. Original magnification 100x. Results are presented as means ± SEM. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ *vs*. RWPE challenged sensitized mice exposed to sham field. $^{###}$ $p < 0.001$, significant difference between Group III and IV.

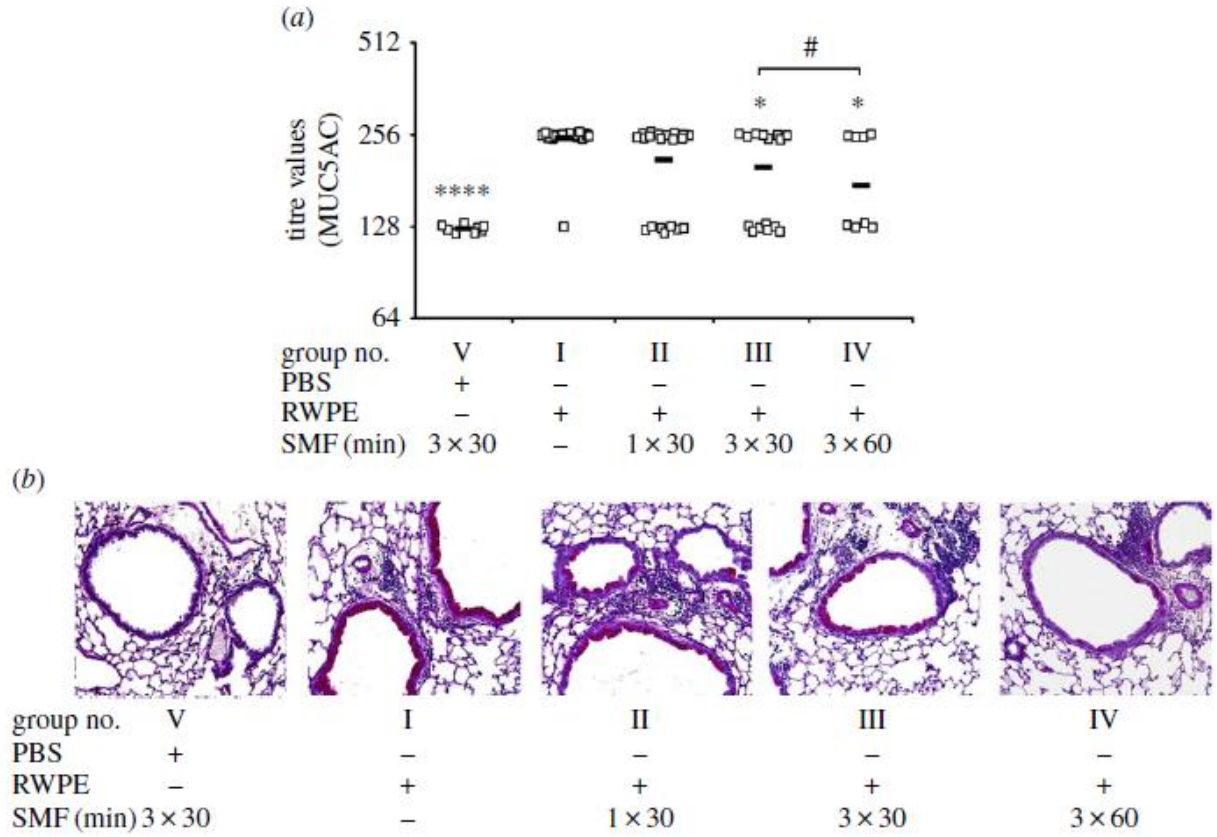

FIGURE 4. SMF-exposure decreases mucin levels and epithelial cell metaplasia in the airways of RWPE-challenged sensitized mice. (**a**) MUC5AC levels in the BAL fluids of RWPE- or PBS-challenged sensitized mice exposed to SMF or sham field. MUC5AC levels were measured by means of ELISA and the results were expressed as endpoint titers (□) and means (-). * $p < 0.05$, **** $p < 0.0001$ *vs*. RWPE challenged, sensitized mice exposed to sham field, $^{\#}$ $p < 0.05$, significant difference between Group III and IV. (**b**) Periodic acid-Schiff staining of formalin fixed lung sections. Original magnification 100x.

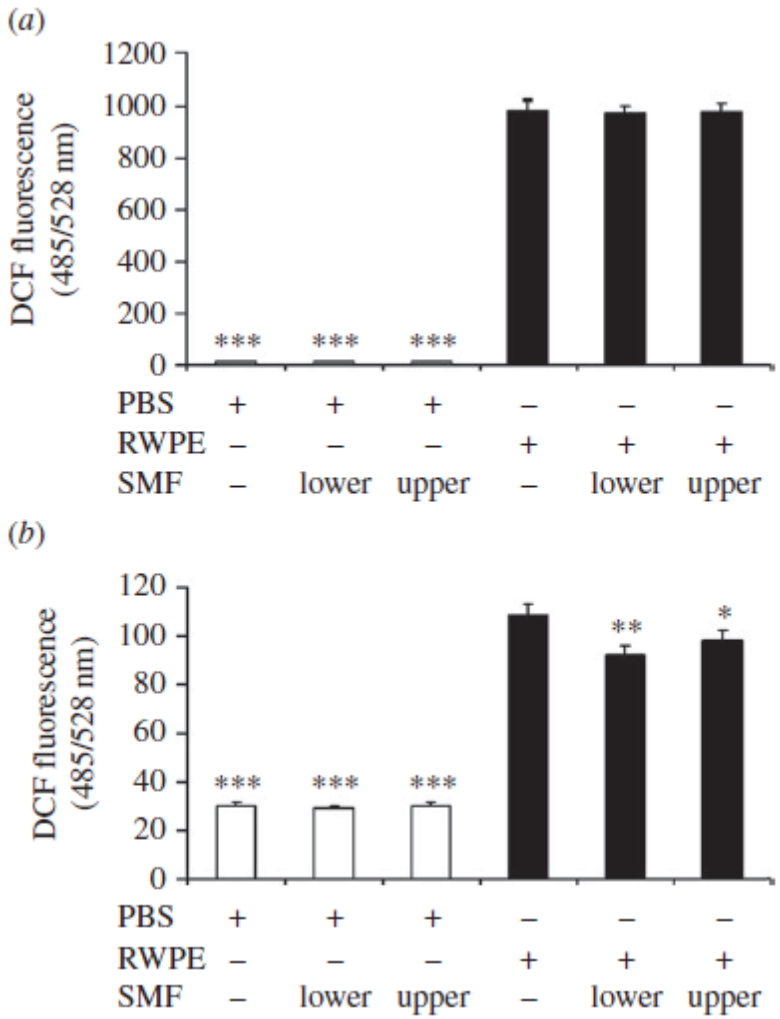

FIGURE 5. SMF-exposure does not alter the generation of ROS by RWPE in cell-free conditions, while diminishes the increase in intracellular ROS levels in RWPE-treated epithelial cells. (**a**) PBS (□) or RWPE (■) solutions containing redox-sensitive H_2DCF-DA were exposed to SMF or sham field for 30 min at lower or upper position (see Material and Methods). Changes in DCF fluorescence intensity were detected by means of fluorimetry. *** $p < 0.001$ *vs.* RWPE exposed to sham field. (**b**) A549 cells loaded with H_2DCF-DA were treated with PBS (□) or RWPE (■) and immediately after the treatment they were exposed to SMF or sham field for 30 min at lower or upper position. Changes in DCF fluorescence intensity are presented as means ± SEM of three

independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs*. RWPE treated cells exposed to sham field.

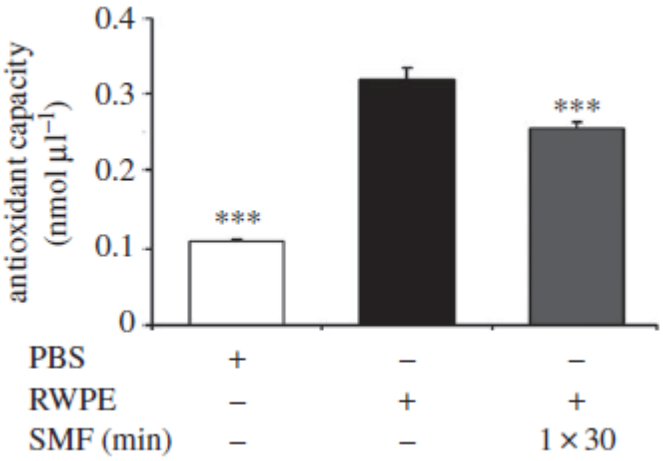

FIGURE 6. SMF-exposure following intranasal challenge lowers the RWPE-induced increase in total antioxidant capacity of the airways. Naive mice were challenged intranasally with RWPE or PBS and immediately thereafter were exposed to SMF or sham field for 30 min. BAL fluid samples were collected 15 min after SMF- or sham field-exposure. Antioxidant potential was measured spectrophotometrically in the supernatant of the samples and expressed in Trolox equivalents. Data are presented as means ± SEM. *** $p < 0.001$ *vs*. RWPE challenged, naive mice exposed to sham field.

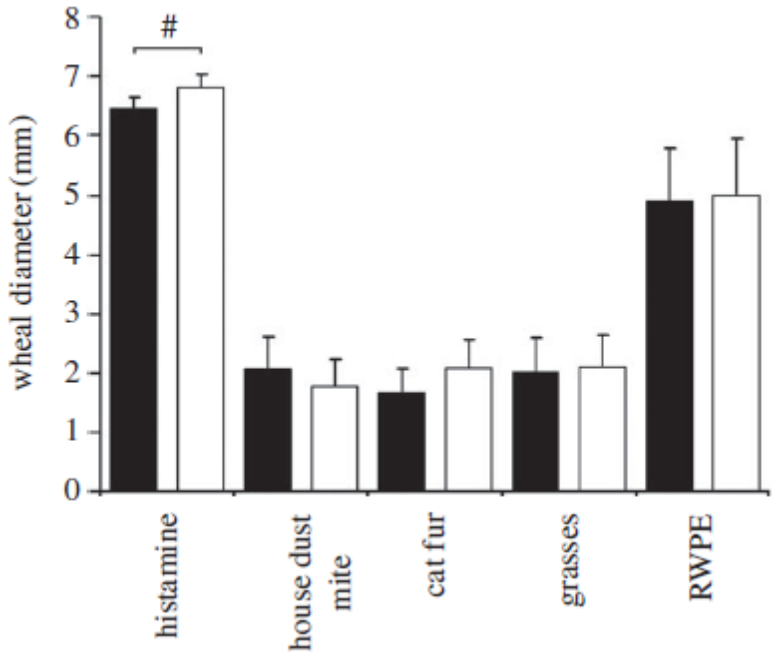

FIGURE 7. SMF-exposure has no significant direct effect on provoked mast cell degranulation. Skin prick tests were performed on healthy volunteers as described in Material and Methods. Immediately after introduction of the identical test materials into the skin of both inner forearms, one forearm of the volunteers was exposed to SMF (■) while the other one was exposed to sham field (□). The wheal reaction was measured immediately after a 15 min exposure period. Data are presented as means ± SEM. $^{\#}p < 0.05$.

Table 1. Ten essential dosing parameters for the present study (as suggested in [18]).

Target tissue	inner forearm skin following provoked skin allergy test
Site of magnet application	both forearms: simultaneous sham-exposure on one and SMF-exposure on the other
Distance of magnet surface from target tissue(s)	3 mm
Magnetic field induction	B_r=1.20 T (remanent induction), 192.28±0.1 mT (peak-to-peak magnetic induction, averaged for all neighbors) by 18.89 T/m lateral gradient (lateral magnetic induction gradient of the main induction component, averaged for all neighbors) at 3 mm from a cylindrical magnet in the isocenter of the matrix along its axis
Material composition of permanent magnet	N35 grade neodymium-iron-boron (NdFeB)
Magnet dimensions	rectangular matrix (140x100 mm) containing 140 pieces of 10x10 mm cylindrical magnets
Magnet polar configuration	neighboring magnets are placed with alternating poles (checkerboard configuration)
Magnet support device	ferrous plate above the magnets, spacers with soft coverage on the contact sites
Frequency of magnet application	single session, continuous exposure
Duration of magnet application	15 min

Short title: Exposure to static magnetic field reduces allergic inflammation